LC/MS/MS identification of 20-hydroxyecdysone in a scorpion (*Liocheles australasiae*) and its binding affinity to *in vitro*-translated molting hormone receptors

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Abstract

Recent advances in mass spectrometry (MS) technology have facilitated the detection and quantification of minor components in organisms and the environment. In this study, we successfully identified 20-hydroxyecdysone (20E) in first instar nymphs (7 days after hatching) of the scorpion *Liocheles australasiae,* using tandem mass spectrometry combined with high-performance liquid chromatography (LC/MS/MS). This substance was not found in adults after the fifth stage. Other possible molting hormone candidates such as makisterone A (MaA) and ponasterone A (PoA), both of which are reported to be the molting hormones of a few arthropod species, were not detected in this scorpion. The ligand–receptor binding of 20E and its analogs was quantitatively evaluated against the *in vitro*-translated molting hormone receptor, the heterodimer of ecdysone receptor (EcR) and the retinoid X receptor (RXR) of *L. australasiae* (LaEcR/LaRXR). The concentrations of ecdysone (E), MaA, 20E, and PoA that are required to inhibit 50% of [^{3}H]PoA binding to the LaEcR/LaRXR complex were determined to be 1.86, 0.69, 0.05, and 0.017 μM, respectively. The activity profiles of these 4 ecdysteroids are consistent with those obtained for the molting hormone receptors of several insects. The binding of a non-steroidal E agonist, tebufenozide, to EcR was not observed even at high concentrations, indicating that the structure of the ligand-binding pocket of LaEcR is not favorable for interaction with tebufenozide.

Keywords

20-hydroxyecdysone, ecdysteroids, scorpion, *Liocheles australasiae*, tebufenozide, EcR

Abbreviations

E, ecdysone; 20E: 20-hydroxyecdysone; PoA, ponasterone A; MaA, makisterone A; PTTH, prothoracicotropic hormone; EcR, ecdysone receptor; USP, ultraspiracle; RXR, retinoid X receptor; LC, liquid chromatography; MS, mass spectrometry

1. Introduction

Scorpions, like other arthropods such as insects and crustaceans, grow by repeatedly molting. Some non-arthropods, such as nematodes, also grow by molting. Although the molting mechanism has been intensively studied in various insects (Henrich, 2005) and crustaceans (Asazuma *et al.*, 2007, Nakatsuji and Sonobe, 2004), little is known about it in other molting animals such as Chelicerates, including scorpions.

Insect molting is triggered by an increase in the titer of 20-hydroxyecdsyone (20E) (Fig. 1) in the hemolymph. In most insects, ecdysone (E) (Fig. 1) is synthesized in the prothoracic glands after prothoracicotropic hormone (PTTH) stimulation and is secreted into the hemolymph, where it is subsequently oxidized to 20E in peripheral tissues such as fat bodies (Gilbert and Warren, 2005). In some species, 3-deoxyecdysone is secreted into the hemolymph, converted to E, and oxidized to 20E. After 20E is transported to the target tissues and organs, it is recognized by the ecdysone receptor (EcR), which activates genes by forming a heterodimer with the ultraspiracle (USP, a homolog of the retinoid X receptor [RXR]) protein (Nakagawa and Henrich, 2009). In some species, the structurally similar ecdysteroids makisterone A (MaA; Fig. 1) (Feldlaufer *et al.*, 1986, Kelly *et al.*, 1984, Tohidi-Esfahani *et al.*, 2011a) and ponasterone A (PoA; Fig. 1) (Lachaise *et al.*, 1986) are utilized as the molting hormones instead of 20E. It has also been reported that E functions as a potent ligand in the mosquito *Aedes aegypti* (Wang *et al.*, 2000) and regulates a distinct set of genes that differs from those controlled by 20E seen in *Drosophila* larval organ culture (Beckstead

et al., 2007).

The genes encoding EcR (Koelle *et al.*, 1991) and USP (Oro *et al.*, 1990) were first identified in *Drosophila*. Furthermore, a number of *EcR* genes have been cloned, primarily from insects (Nakagawa and Henrich, 2009). We recently cloned the *EcR* and *RXR* genes from the scorpion, *Liocheles australasiae*, and denoted the corresponding proteins as LaEcR and LaRXR, respectively (Nakagawa *et al.*, 2007). We also demonstrated that the LaEcR/LaRXR complex specifically binds to ecdysone response elements such as hsp27 and pal1 (Nakagawa *et al.*, 2007). Another study demonstrated that PoA can specifically bind to the LaEcR/LaRXR complex with very high affinity (Nakagawa *et al.*, 2007). Although these findings suggest that scorpions use ecdysteroids as their molting hormone, the structure of the hormone that acts in scorpions has yet to be identified.

Recent advances in spectrometric methods have greatly facilitated the detection of small amounts of chemicals in biological samples. Among various analytical methods, mass spectrometry (MS) is one of the most sensitive analytical systems. In this study, we used the tandem MS technique to detect the natural molting hormone that regulates the growth of scorpions. The binding affinity of several ecdysteroids to the LaEcR/LaRXR complex was evaluated and compared with that in other insect species.

2. Materials and Methods

2.1. Animals

Scorpions were collected on Ishigaki Island, Okinawa Prefecture, Japan, and reared in the laboratory. They were kept in plastic containers with multi-layered wet papers at 25°C and fed live crickets every week. The nymphal stage durations were carefully recorded for scorpions that were individually maintained at 25°C in small plastic cups.

2.2. Chemicals

Ecdysone, 20E, MaA, and PoA were purchased from Fluka (St. Louis, MO), Sigma (St. Louis, MO), A.G. Scientific Inc. (San Diego, CA), and Invitrogen (Carlsbad, CA), respectively. [^{3}H]PoA (140 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Tebufenozide was synthesized in our laboratory (Oikawa *et al.*, 1994).

2.3. Ecdysteroid extraction

First instar nymphs (ca. 7 days old, 107 specimens; 820 mg in total) that were swarmed on the backs of their mothers just before molting were collected and used for the ecdysteroid extraction. Adults after the fifth instar (6 specimens, 1761 mg in total) were also used. The specimens were homogenized in liquid nitrogen with a mortar and pestle, and 4 mL acetonitrile was then added. The homogenate was centrifuged after standing for 24 h at 4°C, and the supernatant was subsequently concentrated by centrifugal evaporation. The extract was loaded on a Sep-Pak C18 Plus cartridge (Waters, Milford, MA), washed with 2 mL of 0.1% aqueous formic acid, and eluted with 2 mL of 70% aqueous acetonitrile to partially purify the extract. The eluate was evaporated to dryness,

re-dissolved in 30 μL of ethanol, and subjected to liquid chromatography (LC)/MS/MS analysis in the selected reaction monitoring (SRM) mode.

2.4. LC/MS/MS analysis

The sample was analyzed in an LC/MS/MS system consisting of an Agilent 1100 HPLC system coupled to an API3000 triple quadrupole mass spectrometer (AB SCIEX; Foster City, CA) equipped with an electrospray ionization source. High-performance liquid chromatography (HPLC) separation was performed on a Cadenza CD-C18 column (75 × 2 mm; Imtakt, Kyoto, Japan) with a 0.2 mL/min flow rate at 30°C by using acetonitrile containing 0.1% acetic acid (A) and 0.1% aqueous acetic acid (B) with a gradient condition of 10% (B) for 1 min and 10–90% (B) for 15 min. The injection volume of the sample was 3 μL. MS/MS analysis was performed under the following conditions: NEB, 14 L/min; CUR, 15 L/min; IS, 4500 V; TEM, 400°C; DP, 30 V; FP, 200 V; EP, 10 V; and CXP, 15 V. SRM analysis was performed using the transitions specific for E, 20E, MaA, and PoA (Table 1). The obtained data were processed using the Analyst 1.3 software (AB SCIEX, Foster City, CA). The concentrations of the compounds in the bodies of the scorpions were estimated using the peak areas of the SRM chromatogram on the basis of a calibration curve constructed using the standards.

2.5. Receptor binding assay

The ligand-binding assay was performed using *in vitro*-translated receptor proteins as described previously (Minakuchi *et al.*, 2003, Ogura *et al.*, 2005). In brief, LaEcR and

LaRXR were prepared using the TNT T7 Coupled Reticulocyte Lysate (Promega, Madison, WI). *In vitro*-translated EcR and RXR were incubated with [^{3}H]PoA (5.55 TBq/mM, 5 nM) for 90 min at 25°C. After incubation, the reaction mixtures were filtered through a GF-75 glass filter (ADVANTEC MFS Inc., Tokyo, Japan). The glass filters were washed and transferred to a vial in a liquid scintillation counter (LSC) with 3 mL Aquasol-2 (PerkinElmer, Waltham, MA) to measure the radioactivity using an LSC-5100 and an LSC-6100 (ALOKA, Tokyo, Japan). The concentrations required for 50% inhibition of [^{3}H]PoA (IC_{50}) binding were determined from each concentration-response curve.

3. Results and Discussion

3.1. Nymphal stage duration

Before we initiated the scorpion ecdysteroid analysis, we examined the molting timing since the molting hormone is detectable just before molting (Gelman *et al.*, 2005, Hsiao *et al.*, 1975, Lachaise *et al.*, 1986, Truman and Riddiford, 1999). Makioka previously examined the period of each nymphal stage of *L. australasiae*; the duration of the first instar nymphs was reported to be about a week (6.7 ± 0.98 days) (Makioka, 1993). In contrast, those of the second and later instars were very long and variable: 76.7 ± 13.31, 52.7 ± 15.19, 167.0 ± 32.61, and 138.6 ± 22.48 days for the second, third, fourth, and fifth instars, respectively. In this study, we recorded the durations of the first, second, and third instars under our rearing conditions. As shown in Table 3, the duration of each stage was relatively longer in our experiments, and the first stage

exhibited the least deviation of duration among the first 3 stages, a finding that is consistent with the results reported by Makioka (Makioka, 1993). Since the durations of the second and later instars varied from specimen to specimen as stated above, we examined the timing of molting from the perspective of the morphological changes of each specimen. We carefully observed the growth of the second and third instars, but we observed no significant signs of molting. Body weight did not significantly change during the second or third instar either (data not shown). Therefore, we decided to extract ecdysteroids from first instar nymphs (7 days old). More than 100 first instar nymph specimens were collected and homogenized for the MS experiments. For comparison, adult specimens after delivery were also submitted for ecdysteroid analysis.

3.2. Ecdysteroid identification in the scorpion hemolymph

Ecdysteroid analysis was performed using LC/MS/MS in the SRM mode (Table 1). The ecdysteroid present in the hemolymph was identified on the basis of corresponding peaks in the SMR chromatograms of the extracted and reference samples. When we applied this method to the identification of E and 20E in the hemolymph of fourth instar larvae of the common cutworm (*Spodoptera litura*), roughly 0.1 and 0.5 μg/mL of E and 20E, respectively, were detected just before the molting. These values are comparable to those reported in other insects as shown below, indicating the validity and reliability of this method. As shown in Fig. 2, 20E was detected in the extract of the first instar nymphs of *L. australasiae* but not in those of adults. Other ecdysteroids such

as E, MaA, and PoA were not detected in either first instar nymphs or adults. The concentration of 20E in first instar nymphs was determined to be 1.1 ng/g body weight via comparison with the peak areas of the standard chromatograms.

Recently, HPLC coupled to LC/MS/MS has been used to identify ecdysteroids and their glycosides in plants such as *Sida rhombifolia* (Wang *et al*., 2008) and *Limnanthes alba* (Stevens *et al*., 2008) as well as in calf urine (Destrez *et al*., 2008). Ecdysteroids are detectable in the range of μg (per liter) using this method, while vertebrate corticoids are detectable in the range of ng (per liter) using micro-LC/MS/MS (Mazzarino and Botre, 2006). Blais et al. used ultra-sensitive nano LC/MS/MS to characterize the ecdysteroids produced by larval ring glands *in vitro* and those present in the third instar larvae of *Drosophila melanogaster* (Blais *et al*., 2010). Based on their analysis, fragments with a loss of 2 water molecules were used for the SRM analysis of E (*m*/*z* 465 > 429), 20E (*m*/*z* 481 > 445), and MaA (*m*/*z* 495 > 459) in this study. In addition, the *m*/*z* of the fragment obtained after the cleavage of the bond between C23 and C24 with the loss of 1 water molecule was also used to specify E (*m*/*z* 465 > 331), 20E (*m*/*z* 481 > 371), and MaA (*m*/*z* 495 > 371). In the case of PoA, a fragment with *m*/*z* = 249, which was possibly produced by the cleavage of 3 bonds (C8–C14, C13–C14, and C13–C15), and a fragment with *m*/*z* = 283 produced by cleavage of the C17–C20 bond with loss of 2 water molecules, were selected for the SRM analysis, to specifically detect PoA.

The titration profile of 20E was previously examined in several insects by using radioimmunoassays. The maximum concentration of 20E for the large titer peak was

estimated to be 1.5 μg/mL in the hemolymph of the last (fifth) instar larva of the tobacco hornworm *Manduca sexta* (Bollenbacher *et al.*, 1981). A similar ecdysteroid titer profile was also recorded in the ovoviviparous cockroach *Nauphoeta cinerea* (Lanzrein *et al.*, 1985), in which the maximum concentrations of 20E and E were estimated to be approximately 3.5 and 0.15 μg/mL, respectively. Since the immunoassay specificity for each ecdysteroid is not high, the values obtained in these studies could have been overestimated. Nishioka et al. determined the optimum concentration of 20E required for the induction of chitin synthesis in the cultured integument of the rice stem borer *Chilo suppressalis* to be 1 μg/mL (Nishioka et al., 1979). Although the concentrations of the 20E titer peaks of insects were thought to be in the range of micrograms (per milliliter), lower concentrations have also been reported for other insect species. The concentration of 20E at its peak is 0.2–0.3 μg/mL for lubber grasshoppers, *Romalea microptera* (Hatle *et al.*, 2003), and 0.2 ng/mL for *D. melanogaster* (Hodgetts *et al.*, 1977, Kraminsky *et al.*, 1980). Ecdysteroids also occur in nematodes such as *Dirofilaria immitis* (Baker and Rees, 1990) and snails such as *Helix pomatia* (Romer, 1979), both of which are categorized as molting animals. The concentration of 20E in *H. pomatia* was determined to be 3.65 ng/g by gas/LC (Romer, 1979), while the concentrations of the free ecdysteroids were quantified to be 1.9–3.9 ng/g wet weight for *D. immitis* by radioimmunoassay (Mendis *et al.*, 1983).

In the present study, we detected 20E in the extract of scorpion nymphs at a concentration of 1.1 ng/g body weight. When the percentage of hemolymph of the total body weight is assumed to be 25% (Gefen and Ar, 2004), the concentration of 20E in

the hemolymph of *L. australasiae* was calculated to be 4.4 ng/mL; this value is significantly lower than that of *M. sexta* and *N. cinerea*. Although the concentration of 20E in *L. australasiae* was roughly estimated, the amount required to activate *L. australasiae* molting might be low, as observed in *D. melanogaster* (Hodgetts *et al.*, 1977, Kraminsky *et al.*, 1980). Alternatively, since the first molting of *L. australasiae* occurs 7–9 days after hatching, the timing of extraction (7 days) may not have exactly matched the highest peak of the 20E titer before molting.

3.3. Binding affinity of ecdysteroids to EcRs

Previously, we successfully cloned the cDNA of the *EcR* and *RXR* genes of *L. australasiae* and prepared their corresponding proteins by using *in vitro* translation systems (Nakagawa *et al.*, 2007). It is well known that [^{3}H]PoA specifically binds to LaEcR with high affinity (K_d = 3 nM) in the absence of RXR. Since the binding assay for insects was performed against an EcR/USP heterodimer, the LaEcR/LaRXR heterodimer was used to determine the affinity of 4 representative ecdysteroids in this study. The concentration-response curve for the inhibition of [^{3}H]PoA binding to the *in vitro*-translated LaEcR/LaRXR heterodimeric proteins by 20E is shown in Fig. 3. The 50% inhibitory concentration (IC_{50}) of 20E was determined to be 0.050 μM, whereas the IC_{50} value of E was 37-fold greater (1.86 μM) than that of 20E. This finding is consistent with previous data obtained using EcR/USP complexes of various insect species belonging to different insect orders (i.e., Lepidoptera, Diptera, Coleoptera, and Hemiptera) (Graham *et al.*, 2007, Minakuchi *et al.*, 2003, Minakuchi *et al.*, 2005,

Minakuchi *et al.*, 2007, Ogura *et al.*, 2005, Tohidi-Esfahani *et al.*, 2011b), according to which E exhibited ca. 13- to 91-fold greater IC_{50} values than those of 20E. The binding activity of 4 ecdysteroids to the LaEcR/LaRXR complex increases in the following order: E, 20E, MaA, and PoA. This finding is also consistent with the results obtained using other insects (Nakagawa and Henrich, 2009). These findings support the hypothesis that 20E acts as the molting hormone in scorpions, although a number of other criteria need to be fulfilled as well (Barrington, 1979). On the other hand, the non-steroidal ecdysone agonist tebufenozide did not significantly inhibit [^{3}H]PoA binding even at high concentrations (100 μM), although it binds to insect EcRs with varying affinities (Table 2). Tebufenozide strongly binds to the EcRs of lepidopteran insects such as *C. suppressalis* (Minakuchi *et al.*, 2003), *Spodoptera frugiperda* (Nakagawa *et al.*, 2000), and *Plodia interpunctella* (Carlson *et al.*, 2001). The binding affinity of tebufenozide to the EcRs of insects in other orders such as *D. melanogaster* (Minakuchi *et al.*, 2005), *Leptinotarsa decemlineata* (Ogura *et al.*, 2005), *Lucilia cuprina* (Graham *et al.*, 2007), and *Nezara viridula* (Tohidi-Esfahani *et al.*, 2011b) is lower than that against lepidopteran insects, although it is comparable to the binding activity of E and a known phytoecdysone (MaA). This finding is probably due to the fact that the structures of the EcR ligand-binding domains required for tebufenozide binding are very different among insect of different orders (Nakagawa *et al.*, 2007) and arthropods. It is also known that the binding affinity of tebufenozide is negatively correlated with the distance in the phylogenetic trees constructed for the EcR ligand-binding domains (Graham *et al.*, 2009). The sequence similarity of the EcR

ligand-binding domains between *L. australasiae* and *C. suppressalis* is only 54%, suggesting that the EcR of *L. australasiae* cannot accommodate tebufenozide in its ligand-binding pocket. This information may be useful for examination of the ligand–receptor binding mode and the development of other selective bioactive compounds.

Acknowledgements

This study was supported in part by the 21st century COE program for Innovative Food and Environmental Studies Pioneered by Entomomimetic Sciences of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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Figure Legends

Fig. 1 Ecdysteroidal structures

Fig. 2 Selected reaction monitoring (SRM) chromatograms for 20E (A), E (B), PoA (C), and MaA (D). For each ecdysteroid, standard (0.3 pmol), nymph extract, and adult extract data are shown in the upper trace. 20E: 20-hydroxyecdysone; E, ecdysone; PoA, ponasterone A; MaA, makisterone A.

Fig. 3 Concentration-response curve for the inhibition of [^{3}H]ponasterone A binding to the *in vitro*-translated heterodimer of the ecdysone receptor and the retinoid X receptor of *L. australasiae* by 20-hydroxyecdysone.

Table 1. SRM conditions used in tandem mass spectrometry analysis

Ecdysteroid	Retention time	SRM transition	CE (V)
E	6.7	465 > 429	20
		465 > 331	20
20E	6.0	481 > 445	20
		481 > 371	20
MaA	6.5	495 > 459	25
		495 > 371	30
PoA	8.3	465 > 283	30
		465 > 249	30

SRM, selected reaction monitoring; CE, collision energy; E, ecdysone; 20E: 20-hydroxyecdysone; MaA, makisterone A; PoA, ponasterone A

Table 2. Binding activity of various ecdysone agonists to 4 *in vitro*-translated EcR/USP (RXR) heterodimer proteins

	Liocheles australasiae		*Chilo suppressalis*[a]		*Drosophila melanogaster*[b]		*Leptinotarsa decemlineata*[c]	
	IC_{50} (μM)	K_i (μM)[d]	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)	IC_{50} (μM)	K_i (μM)
20E	0.050	0.020	0.22	0.042	0.093	0.014	0.44	0.16
E	1.9	0.73	20.0	3.9	5.8	0.84	10	3.8
MaA	0.69	0.27	0.47	0.091	0.14	0.020	1.8	0.65
PoA	0.017	0.0066	0.0080	0.0015	0.0050	0.00070	0.0070	0.0025
Tebufenozide	>100 (16%)[e]	39.0	0.0010	0.00020	0.98	0.14	6.6	2.4

EcR, ecdysone receptor; USP, ultraspiracle; RXR, retinoid X receptor; 20E: 20-hydroxyecdysone; E, ecdysone; MaA, makisterone A; PoA, ponasterone A

a) Minakuchi *et al.*, 2003

b) Minakuchi *et al.*, 2005

c) Ogura *et al.*, 2005

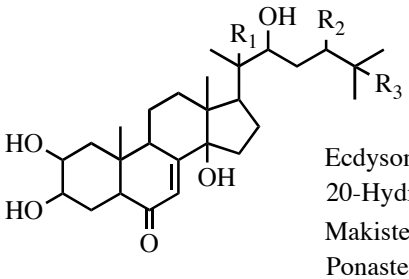
d) The K_i values were calculated using the equation $K_i = IC_{50}/(L/K_d+1)$, where L is the [^{3}H]PoA concentration used for the binding assay and K_d is the radioligand equilibration dissociation constant (Nakagawa *et al.*, 2007).

e) Percentage inhibition of the [^{3}H]PoA binding with 100 μM tebufenozide. The standard deviation of inhibition is 10% (n = 6).

Table 3. Nymphal stage durations

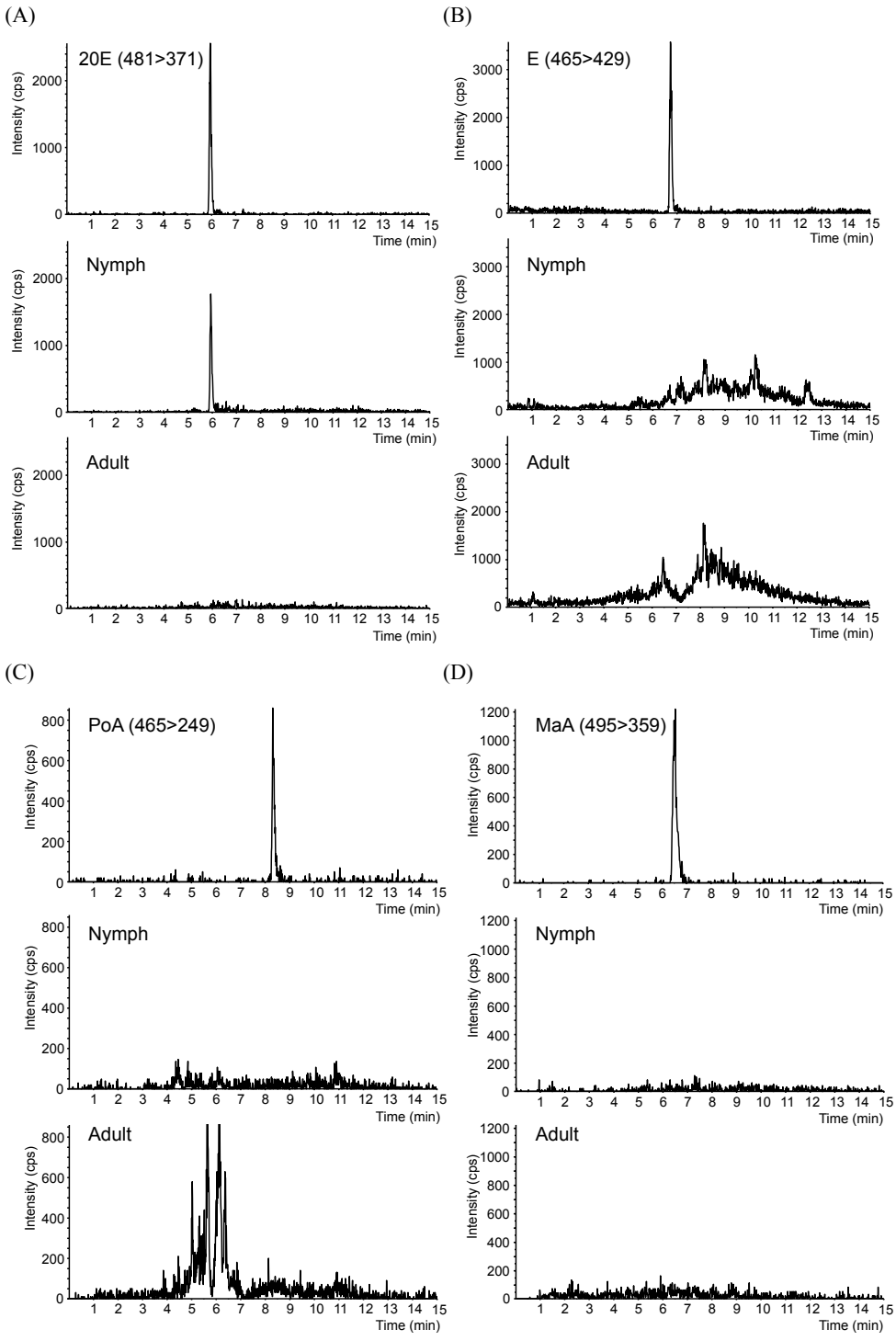
Nymph stage	Days[a)]
1	7.9 ± 0.4 (n = 36)
2	91.9 ± 25.6 (n = 79)
3	90.6 ± 26.2 (n = 39)

[a)] n, the number of specimens used for observation



Ecdysone (R_1=R_2=H, R_3=OH)
20-Hydroxycdysone (R_1=OH, R_2=H, R_3=OH)
Makisterone (R_1=OH, R_2=CH_3, R_3=OH)
Ponasterone A (R_1=OH, R_2=R_3=H)

Fig. 1 Ecdysteroidal structures



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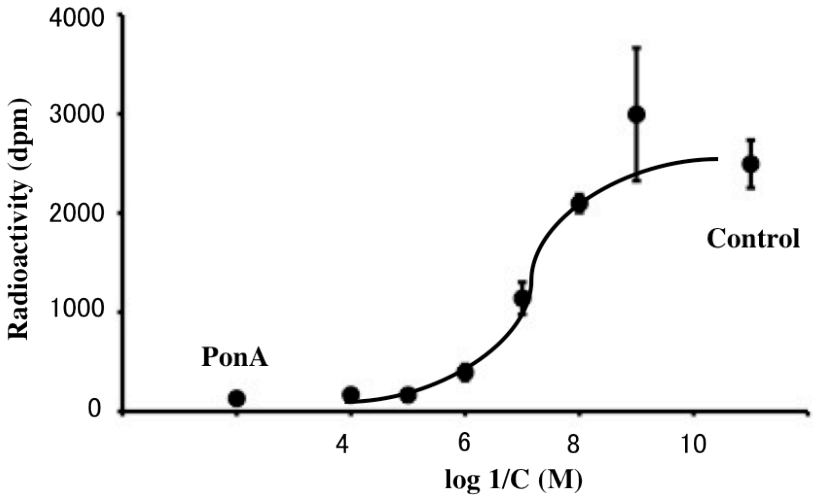

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